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# 1 **Re-annotation of the genome sequence of *Clostridium difficile* strain 630**

2 Marc Monot<sup>1</sup>, Caroline Boursaux-Eude<sup>2</sup>, Marie Thibonnier<sup>1</sup>, David Vallenet<sup>3</sup>, Ivan  
3 Moszer<sup>2</sup>, Claudine Medigue<sup>3</sup>, Isabelle Martin-Verstraete<sup>1,4</sup> and Bruno Dupuy<sup>1</sup>

4 <sup>1</sup> Institut Pasteur, Laboratoire Pathogénèse des Bactéries Anaérobies, 28 rue du Dr Roux, 75724 Paris Cedex  
5 15, France

6 <sup>2</sup> Institut Pasteur, Intégration et Analyse Génomiques, 28 rue du Dr Roux, 75724 Paris Cedex 15, France

7 <sup>3</sup> CNRS UMR 8030, Université d'Evry & CEA, IG, Genoscope - LABGeM - 2 rue Gaston Crémieux, CP5706, F-  
8 91057 Evry cedex, France

9 <sup>4</sup> Université Paris Diderot-Paris 7 – 7 boulevard Diderot, 75012 Paris, France

## 11 **Correspondence**

12 Marc Monot

13 [mmonot@pasteur.fr](mailto:mmonot@pasteur.fr)

14 Tel. +331 45 68 83 16 / Fax. +331 40 61 31 83

16 A regular update of genome annotations is a prerequisite step to help maintain the  
17 accuracy and relevance of the information they contain. Five years after the first  
18 publication of the complete genome sequence of *C. difficile* strain 630, we manually re-  
19 annotated each of the coding sequences (CDS), using a high-level annotation platform.  
20 The function of more than 500 genes annotated previously with putative functions, were  
21 re-annotated based on updated sequence similarities of proteins whose functions have  
22 been recently identified by experimental data from the literature. We also modified 222  
23 CDS starts, detected 127 new CDS and added the enzyme commission numbers, which  
24 were not supplemented in the original annotation. In addition, an intensive project was  
25 undertaken to standardise the names of genes and gene products and thus harmonising  
26 as much as possible with the HAMAP project. The re-annotation is stored in a relational  
27 database that will be available on the MicroScope web-based platform,  
28 “<http://www.genoscope.cns.fr/agc/microscope/ClostridioScope>”. The original  
29 submission stored in the INSDC nucleotide sequence databases was also updated.

31 **Scope :** *Clostridium difficile* special issue of the *Journal of Medical Microbiology*

33 \*The EMBL accession number for the re-annotation of *C. difficile* strain 630 is AM180355  
34 and its plasmid pCD630 is AM180356.

## INTRODUCTION

The re-annotation of several model genomes has been recently performed, among these there are *Escherichia coli* for the gram negative bacteria (Luo *et al.*, 2009) and *Bacillus subtilis* for the firmicutes (Barbe *et al.*, 2009). This provided new information about genomic structure and organisation as well as gene function and plays an essential role in defining reference knowledge. In addition the re-annotation of the *B. subtilis* genome also benefits the other firmicutes such as *Clostridium difficile*.

*C. difficile* is one of the major enteropathogenic clostridia and *C. difficile*-associated diarrhea (CDAD) is currently the most frequently occurring nosocomial infection in many European hospitals. Although toxins are generally recognised as the main virulence factors, *C. difficile* pathogenesis remains poorly understood. The global genetic analysis of *C. difficile* appeared to be an useful approach to find potential mechanisms involved in the bacterial virulence for which an updated of the gene list and corresponding annotations is tremendously important.

The first complete genome sequence of a *C. difficile* strain (630) was sequenced in 2006 (Sebahia *et al.*, 2006). It led to the development of high throughput projects such as comparative genomic, transcriptomic and proteomic studies (Jain *et al.*, 2010; Janvilisri *et al.*, 2010; Marsden *et al.*, 2010), which were recently reinforced with an increase of multiple genomic projects (Stabler *et al.*, 2009). However, the relevance of all these experiments greatly depends on the information available for the genes particularly their functions experimentally identified or predicted *in silico*. Thus, it is critical that the information is accurate, relevant and useful. This is why we undertook the re-annotation of the *C. difficile* strain 630 genome.

The advances in second-generation sequencing technologies combined with their relative low cost has led to the increased need for a rapid genome annotation system (Petty, 2010). However the fastest way to obtain an accurate annotation remains to transfer annotation from a reference strain. This requires to have access to a closely related genome for each species annotated to a high standard and regularly updated.

We described in this paper the manual re-annotation of all CDS of the *C. difficile* strain 630 genome. For this purpose we used improved methods in bioinformatics, literature surveys and genome data from closely related species such as *Clostridium sticklandii*, which has recently been sequenced (Fonknechten *et al.*, 2010) or *B. subtilis* whose genome has been re-sequenced and re-annotated (Barbe *et al.*, 2009). The re-annotation resulted in the function precision of more than 500 genes and the addition of new CDSs as well as the correction of the start sites of 222 CDSs. All information from laboratory research publications could be continuously integrated through the MicroScope platform to maintain this up-to-date annotation.

## METHODS

### Identification of new or modified CDS in the *C. difficile* genome

The sequence and the original annotation of the published *C. difficile* 630 genome (Sebahia *et al.*, 2006) was integrated into the Microscope platform (Vallenet *et al.*, 2009). MicroScope is a web-based framework for the systematic and efficient revision of microbial genome annotation and comparative analysis. Its main features are (i) integration of annotation data from bacterial genomes enhanced by a gene coding re-annotation process using accurate gene models, (ii) integration of results obtained with a wide range of bioinformatics methods, among which exploration of gene context by searching for conserved synteny and reconstruction of metabolic pathways, (iii) an advanced web interface allowing multiple users to refine the automatic assignment of gene product functions. MaGe is also linked to numerous well-known biological databases and systems. The original gene prediction was systematically checked using the AMIGene software (Bocs *et al.*, 2003) and the MICheck strategy (Cruveiller *et al.*, 2005). The initial identifier of genes 'CD0000(A)' used a prefix of two letters, 'CD', followed by a four-digit number corresponding to the position of CDS in the genome. Whenever a new gene interleave, a capital letter was added in alphabetical order. Since 2006, the locus tag usage has evolved (Cochrane *et al.*, 2008). The prefix now has to contain only alpha-numeric characters and it must be at least 3 characters long. In addition the locus tag prefix must be separated from the tag value by an underscore

ending with a number. So we assigned for all CDS a new locus tag code: 'CD630\_00000'. The four-digit number after the underscore is still the original CDS position in the genome. The capital letter of the original identifier was converted to a number which has been added at the end of each gene : 1 to 9 for genes previously ended with capital letter A to I, and 0 for all others e.g. CD0001 into CD630\_00010 and CD0163B into CD630\_01632. Finally, because the genomic position of the non coding CDS was defined with only three-digit numbers, we replaced the first number after the locus tag prefix with a 't' or 'r' respectively for transfer RNA and ribosomal RNA respectively, e.g. CDt001 into CD630\_t0010 and CDr001 into CD630\_r0010. We used the same coding method for the 11 CDSs encoded by the plasmid pCD630, adding the letter 'p', after the locus tag e.g. CDP01 into CD630\_p010.

During the re-annotation process using the AMIGene predictions, we identified new CDS and we assigned them the locus tag of the previous CDS with the last number incremented by 1 e.g. a new gene detected after CD630\_02670 (previously named CD0267) was coded CD630\_02671. The original locus tag will be kept in the EMBL file using the /Old\_locus\_tag identifier.

#### Re-annotation of the complete *C. difficile* strain 630

The predicted proteins were subjected to a wide range of bioinformatics tools, which includes conserved synteny computations, alignments against TrEMBL and SWISS-PROT databases (Apweiler R, 2011) and TMHMM (Sonnhammer *et al.*, 1998), SignalP (Bendtsen *et al.*, 2004) and PsortB (Yu *et al.*, 2010) software to predict subcellular localization of proteins as well as INTERPROSCAN (Zdobnov & Apweiler, 2001) to identify possible functions of newly discovered proteins (Apweiler R, 2011). This work flow led to an automatic functional annotation for each CDS as previously described (Vallenet *et al.*, 2006). Finally, these pre-computed results served as basis for the manual re-annotation of each CDS proceeding by inference.

To normalise the process of manual annotation among multiple users, we set up several guidelines: (a) The product field is filled with the functional annotation for all genes identified with 'Hypothetical protein' or 'Conserved Hypothetical protein' when the gene was not identified. For all others we added 'Putative' prior to the product

annotation. Pseudogene and gene remnant have a specific nomenclature : "Fragment of" + function + position (N-terminal, C-terminal or center of the encoding protein). (b) The name of gene was completed by searching in the literature using PubMed data libraries (<http://www.ncbi.nlm.nih.gov/pubmed>) and when we changed gene names, old names were indicated in the synonymous field. (c) The start sites were modified according to the combination of the graphical data such as codage probability curves deduced from the AMIGene method (Bocs *et al.*, 2003), as well as alignments with orthologous genes (Altschul *et al.*, 1990). Then, the label '/START=' was added in the comment field followed by a capital letter associated to an informative code (M: modified, C: coding curve, S: sequence similarity, O : overlap, R : RBS). (d) PubMed identifiers (PMIDs) of each gene were classified from the specific references to the articles corresponding to orthologous genes and/or the global reviews concerning its function. (e) Protein families were standardized using the same keywords, PMIDs and global classification, such as CMR roles (<http://cmr.jcvi.org/cgi-bin/CMR/RoleIds.cgi>).

## RESULTS & DISCUSSION

### Evaluation of annotation improvement

The original annotation of the *C. difficile* strain 630, published in 2006 (Sebaihia *et al.*, 2006), identified 3776 predicted coding sequences (CDSs). We have updated annotation of all CDSs and assigned or precised their functions. During the re-annotation process we attributed a class of function to each gene re-annotated: (i) "known": when function was experimentally demonstrated or when high level of similarities with characterised genes were found (ii) "putative": based on conserved motif, structural feature or limited similarities, (iii) "unknown": when genes were unidentified and (iv) "pseudo": for pseudogenes or gene remnants. The same classification was applied manually to the 2006 annotation to allow comparison of both annotations (Table 1A).

Thus, 518 and 18 genes whose encoding function was previously described as putative and unknown respectively have now a functional annotation identified by the experimental data from the literature (Table 1A). For example, CD630\_26030 (previously named CD2603), recognised as a putative response regulator, is now

designated *cdtR*, since it was shown that it controls the binary toxin expression in *C. difficile* (Carter *et al.*, 2007). In addition, 117 genes of unknown function have now a putative function. For instance, 12 conserved hypothetical proteins which contain a CRISPR-associated domain (clustered regularly interspaced short palindromic repeats) are annotated “Putative CRISPR-associated family protein”. Furthermore, we showed that the ATP synthase epsilon chain, CD630\_34670 (CD3467), which was defined as a gene remnant (pseudo class) because of a lack of amino-acid in the C-terminus relative to database matches, actually belongs to the class of “known function”. This enzyme usually combines ATP synthesis and hydrolysis but the hydrolysis function is still active in the truncated version (Ferguson *et al.*, 2006).

Following the re-annotation we included 127 new CDSs and defined 222 new CDS start sites. The majority of the new CDS are divided into putative (25), unknown (86) or pseudogene (15) classes (Table 1B). Only one gene, *CD630\_15951* has an orthologue, whose function was experimentally demonstrated. This gene, detected during the proteomic analysis recently performed in *C. difficile* (Lawley *et al.*, 2009), is highly homologous (~60%) to a ferredoxin gene of *Clostridium thermoaceticum* (Elliott *et al.*, 1982).

We were looking for papers corresponding to each gene, and particularly those published after the original annotation. We added at least one PMID reference number to 64% of the *C. difficile* genes. Like many other genome-wide updates, several specificities were added to the original product function. When possible, we attached new motifs and enzymatic domains identified by INTERPROSCAN, allowing a more accurate description of the original function. For example, putative peptidase enzymes have now family information according to the classification scheme of the MEROPS database (<http://merops.sanger.ac.uk>). The revised nomenclature of the pathogenicity locus region (Rupnik *et al.*, 2005) has been introduced during re-annotation process as well as genes involved in the *C. difficile* motility and flagellar glycosylation since they were recently published (Twine *et al.*, 2009). A locus tag, product annotation and class comparison between the two annotations performed in 2006 and 2010 were summarised in the Table S1. All information of the CDS re-annotated (Fig. S1), are currently available on the MicroScope platform:

<http://www.genoscope.cns.fr/agc/microscope/ClostridioScope>. We also updated the *C. difficile* 630 genomic entry in genomic databases: EMBL, GenBank and DDBJ.

### Deciphering the annotation origin

Several pieces of information appeared when we evaluated the source used during the functional annotation of known, putative or unknown genes (Fig. 1). In the known category, 1% of the gene function came from *C. difficile* strain 630 publications, 1,5% from other *C. difficile* strains, 4% from other clostridia and 93,5% from other species (Fig. 1A.). The putative category was defined according to the enzymatic domain (40%), homology to mobile elements (20%) or cell localisation (15%) (Fig. 1B). As an example a gene will be annotated "Putative membrane protein" when 3 or more transmembrane helix was detected by TMHMM (Sonnhammer *et al.*, 1998). Finally, we classified the unknown genes from the alignment results with TREMBL (Boeckmann *et al.*, 2003). Although 45% were orphan of the *C. difficile* strains, 20% were also found in the genus *Clostridium*, 15% in the firmicutes phylum and 20% in other bacteria (Fig. 1C).

Concerning genes annotated as known, we noted that only few of them came from a published clostridial experiments (Fig. 1A). This was mainly due to the lack of effective tools to mutate clostridial genes. However gene inactivation method and random mutagenesis system recently developed in *C. difficile* (Cartman & Minton, 2010), should greatly improve the number of publications on *C. difficile* gene functions. Half of the genes with an unidentified function, orphan, are found only in *C. difficile* 630 (Fig. 1C.). However, most orphans are present in the *C. difficile* strains already sequenced such as strains 027, CD196 and R20291 (Stabler *et al.*, 2009). This may constitute a source of gene targets that could be used both in research, diagnosis or treatment of the CDAD.

### Miscellaneous improvements

To re-annotate the *C. difficile* genome of strain 630 we used the MaGe interface, which contains classic database fields (type, position, name, product, EC numbers) and several specific fields such as gene synonymous (synonyms), authors notes (comments), pubmed identifiers (PMID), product type, localisation and functional classification (Fig.



S1). All information found during the re-annotation process that did not fit in the classic fields were added in the specific MaGe field or in the comments. For example, the novel virulence factor called Srl for « Sensitivity regulation of *C. difficile* toxins », (Miura *et al.*, 2010) was presented during the third international clostridium difficile symposium. This information was only indicated in the comment field of the CD630\_22980 (CD2298) gene until further validation.

The names of gene products were harmonizing as much as possible with the HAMAP project (Lima *et al.*, 2009). On the other hand, all gene products have now been named with a specific keyword related to their functional family (Fig. S1). Thus, CD630\_05310 (CD0531), previously annotated «DeoR-like regulator of transcription» (a regulator of sugar and nucleoside metabolic systems) was re-annotated « Transcriptional regulator (keyword), DeoR family ». We also normalised the annotation of genes that share the same characteristics. As an example, proteins that were only determined according to their membrane localization were annotated: « Putative membrane proteins ». The annotation standardization we used will facilitate the mining of the data using bioinformatics as well as manual search (Fig. S1).

### Membrane Transport

The *C. difficile* genome contains a lot of proteins encoding several membrane transport systems: ATP-binding cassette (ABC) transporters, phosphoenolpyruvate-dependent phosphotransferase systems (PTS), charged substrate transporters (antiporters, symporters) and facilitators. The general function of the genes encoding such proteins can be easily determined from bioinformatic approaches, like those used for the protein domain analysis in InterProScan (Zdobnov & Apweiler, 2001). However it is quite difficult to distinguish the exact metabolite they transport, especially when the transport systems have a wide specificity. We reannotated most of the transporter systems by inference including clues about targets using specialized databases such as TransportDB (<http://www.membranetransport.org/>)(Ren *et al.*, 2007) which compile all information on cytoplasmic membrane transporters. We added a suffix in the classification which indicate, from a global trend to the expected target, the motif (family), the high sequence homology (like) and the evidence of a target metabolite

(specific). However, this classification should be taken with caution since it was mainly deduced from *in silico* analysis rather than from experimental data.

The table 2 showed annotation of 19 PTS systems with a specific metabolite suggestion. The targeted metabolite was deduced from the INTERPROSCAN motif search but could also be defined by the presence in the same locus of gene encoding enzyme involved in specific sugar assimilation (associated enzyme). As an example *CD630\_22690 (CD2269)* is now annotated as “PTS system, fructose-specific IIABC component”. This is due to the detection of three motif signatures the mannitol family PTS EII component A, B and C, as well as the presence of the neighbouring gene, the *CD630\_22700 (CD2270)*, which encodes an enzyme involved in the utilization of fructose: “Fructose 1-phosphate kinase” as indicated in the gene annotation (Table S1).

#### Metabolism update

Updating the genome annotation of *C. difficile* led to many changes within the metabolism pathways. The gene cluster involved in the anaerobic oxidative degradation of L-ornithine has been identified in *C. sticklandii* (Fonknechten *et al.*, 2009). From this publication we reannotated genes *CD630\_04420 (CD0442)* to *CD630\_04480 (CD0448)* whose encoding proteins share high similarities to the ornithine catabolism compounds of *C. sticklandii* e.g. Ord, OrtA, OrtB, OraS, OraE, Or-4 and Orr, respectively (Table S1). This suggested that *C. difficile* could produce acetyl-CoA from the ornithine fermentation. The ability to use a variety of carbohydrates is an important feature for *C. difficile* to colonize the host gut. *Enterococcus faecalis* found in the same niche as *C. difficile*, provided hints to explore the consistency of a specific pathway required for ethanolamine utilisation, a constituent of an abundant class of phospholipids present in the eucaryotic cell membranes and the host’s dietary intake (Del Papa & Perego, 2008) (Fox *et al.*, 2009). Using the *E. faecalis* gene synteny and protein similarities, we were able to reconstruct the whole ethanolamine pathway in *C. difficile*, a cluster of 19 genes, from *CD630\_19060 (CD1906)* to *CD630\_19260 (CD1926)* encoding the ethanolamine ammonia-lyase, an alcohol dehydrogenase, a carboxysome associated proteins, the transporter EutH and the two-component system EutV, EutW. (Table S1).

Interestingly, in *B. subtilis* several enzymes involved in RNA degradation were recently identified (Even *et al.*, 2005) (Shahbadian *et al.*, 2009). In *C. difficile*, a unique Rnase J protein *CD630\_12890* (*CD1289*) was detected as well as an ortholog of *ymdA* *CD630\_13290* (*CD1329*), encoding the Rnase Y protein.

## CONCLUSION

Finally, nearly half of the genes of the *C. difficile* strain 630 encode proteins with known function, whereas one-third of the gene products have a putative function and only fifteen percent of proteins with unknown function are encoded by *C. difficile* genome (Table 1A). In addition, 127 new CDSs were discovered (Table 1B) and 222 CDS starts were modified. The re-annotation was performed using a high standard annotation MicroScope platform, which significantly increased the amount of information available for the majority of the CDS, such as literature references, product types, localisation and gene synonymous (Fig. S1).

Nevertheless, there is still great deal of work to be completed since only 116 annotated genes came from a published clostridial experiments. The EMBL entries are now resubmitted and to maintain the annotation up-to-date, all new information would be addressed directly to marc.monot@pasteur.

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## AUTHORS' CONTRIBUTION

BD, MM, CB-E and IM designed the study. CB-E and MM carried out the major part of the manual re-annotation of the genome together with MT and IM-V. DV and CM were involved in automatic re-annotation and administration of the MicroScope platform. MM, IM-V and BD wrote the manuscript.

## TABLE & FIGURE LEGENDS

Table 1 : Review of the 2006's annotation update. A) CDSs were identified and separated according to the four major annotation classes in both 2006 and 2010 annotations: **(Known)** when function were experimentally demonstrated, **(Putative)** based on conserved motif, structural feature or limited homology, **(Unknown)** when function are unidentified and **(Pseudo)** for pseudogenes. Dark grey padding numbers indicated no change and “±” and “-” correspond to a change between the classes of annotation between 2006 annotation and 2010 re-annotation. B) Annotation of the new CDS detected and referenced as known, putative, unknown and pseudo classes.

Table 2 : Re-annotation of the PTS systems according to the metabolite specificity. List of locus tags corresponding to 19 PTS re-annotated. The PTS metabolite was deduced from the motif class detection and/or the presence of associated enzymes involved in a specific sugar metabolism.

Figure 1 : Distribution of the functional re-annotation origin. A) **Known** functions were identified from the literature references of: *Clostridium difficile* strain 630 (C. diff 630), other *Clostridium difficile* strains (C. difficile), *Clostridium* species (Clostridia) and others species (Others). B) **Putative** functions were defined from: enzymatic domains (Enzyme), homology with mobile elements (Mobile), localisation in the cell (Localization) and the remaining origin (Others). C) **Unknown** functions which were found only in: *Clostridium difficile* (Orphan), in the *Clostridium* species (Clostridia), in the firmicutes phylum (Firmicutes) or in diverse bacteria (Others).

## SUPPLEMENTARY DATA

Table S1 : Comparison between 2006 and 2010 annotations. For each CDS, the locus tags, annotation function and classes are compared for both 2006 and 2010 annotations.

Table S2 : Standardization of family product names. The product names were constructed around a keyword specific to the gene's functional family.

Figure S1 : *C. difficile* CDS re-annotation by MaGe. MaGe annotation window for *tcdR* gene. Bold and grey outline focus on information added specifically within this process : mutation, synonyms, comments, PMID, product type, localization, MaGe classification and standard classification (Bioprocess and Roles).

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Table 1

**A**

<div> <div>2010</div> <div>2006</div> </div>	Known 47%	Putative 37%	Unknown 14%	Pseudo 2%
Known 34%	1222	-63	-0	-10
Putative 47%	+518	1163	-115	-12
Unknown 17%	+18	+177	397	-7
Pseudo 2%	+1	+2	+1	63

**B**

New CDS	Known	Putative	Unknown	Pseudo
127	1	25	86	15



Table 2

Locus tag	Motif class	Associated enzyme	Proposed PTS metabolites
CD630_04690	Glucose	CD630_04680	Sucrose
CD630_03880	Glucose	CD630_03890	b-glucoside
CD630_30970	Glucose	CD630_30950 / CD630_30960	b-glucoside
CD630_31160	Glucose		b-glucoside
CD630_31250	Glucose		b-glucoside
CD630_31370	Glucose		b-glucoside
CD630_26660 / CD630_26670	Glucose		Glucose
CD630_30580 / CD630_30610	Glucose	CD630_30600	a-glucoside
CD630_22690	Mannitol	CD630_22700	Fructose
CD630_30750	Mannitol	CD630_30740	Tagatose
CD630_30860	Mannitol	CD630_30850	2-O-a-mannosyl-D-glycerate
CD630_23320 / CD630_23330	Mannitol	CD630_23310	
CD630_00410 / CD630_00420 / CD630_00430	Mannitol	-	Mannitol
CD630_36450 / CD630_36470 / CD630_36480	Lactose	-	Galactitol
CD630_28800 / CD630_28830 / CD630_28840	Lactose	CD630_28820	Lichenan
CD630_30130 / CD630_30140 / CD630_3015	Mannose	CD630_30120	Cellobiose
CD630_25660 / CD630_25670 / CD630_25680	Mannose	CD630_25690	Mannose
CD630_30670 / CD630_30680 / CD630_30690 / CD630_30700	Mannose	CD630_30710	Mannose
CD630_07640 / CD630_07650 / CD630_07660 / CD630_07670	Sorbitol	CD630_07680	Xyloside
			Sorbitol